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14. ABSTRACT

Our objective was to develop a realistic preclinical model of prostate cancer by developingmethodology that supports the survival, growth and differentiation of primary cultures ofprostate cells in mice. Use of a novel substrate, "OptiCell" membranes, for implantation,choice of the renal capsule for implantation, and selection of stem-like cells forimplantation did not resolve the unwanted development of squamous differentiation. Therefore, we investigated the implantation of precision-cut tissue sections. These tissueslices can be maintained in culture, and our initial results suggest that they also surviveand maintain structure and function under the renal capsule. We will continue to developtissue slices as a novel in vitro – in vivo experimental model of prostate cancer.

15. SUBJECT TERMS
in vivo model development, primary cultures, epithelial and stromal cells, preclinical model,stem cells

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INTRODUCTION

Currently available models of prostate cancer do not realistically predict activity of experimental therapeutic agents in clinical trials. The objective of our proposed research was to develop a model system that will allow the translation of *in vitro* results to an *in vivo* environment and provide a more realistic preclinical model of prostate cancer than currently exists. Primary cultures, which provide a key *in vitro* model of normal and malignant prostate biology, could fulfill this objective if we devised a means by which they could be maintained *in vivo* and express appropriate structural and functional differentiation. Our past studies showed that primary cultures transplanted into *nude* mice via standard subcutaneous injection methods rapidly became squamous. We hypothesized that hypoxia is the factor that triggers inappropriate squamous formation that prevents appropriate growth and prostate-specific differentiation of primary cultures *in vivo*. Our experimental plan was to circumvent hypoxia by transplanting cells on a unique gas permeable membrane under the highly vascularized subrenal capsule of the mouse. Our aims were (1) To transplant primary epithelial cell cultures grown on OptiCell™ membranes under the renal capsule of *nude* mice, (2) to transplant primary stromal cell cultures grown on OptiCell™ membranes under the renal capsule of *nude* mice, and (3) to transplant co-cultures of epithelial and stromal cells on OptiCell™ membranes under the renal capsule of nude mice.

We carried out all of the Aims in Year One, as described in the first annual progress report (February, 2005). However, we encountered unforeseen problems related to the nature of the OptiCell™ membranes. Although primary cultures grew well on the membranes, we found that the membranes were too rigid and provoked a very strong inflammatory and scarring response due to irritation when implanted under the kidney capsule. We concluded that we would have to identify a matrix other than OptiCell™ membranes on which to transplant primary cultures into mice. However, as we developed our experimental strategy for Year Two, we also concluded that we could not ignore the developing field of cancer stem cells. Cancer stem cell theory posits that a rare population of functionally distinct cancer cells possesses the extensive self-renewal potential necessary to create a tumor; these are cancer stem cells (CSCs) [1]. Accordingly, we thought that our original hypothesis, that hypoxia is the factor limiting growth of primary cultures *in vivo*, was naive. While hypoxia may indeed be a critical inhibitory element in the microenvironment, it is perhaps also true that primary cultures will not grow and develop appropriate structure and function *in vivo* unless they contain stem cells. Therefore, in Year Two, we devoted our studies to determining whether our primary cultures as historically established contain a subpopulation of stem cells and, if not, to identifying conditions that permit establishment and growth of stem cells in primary cultures. Our results were described in the Year 2 (February, 2006) progress report. Briefly, we concluded that primary cultures as routinely established did not contain stem cells, but instead consisted of transit amplifying cells (i.e., proliferative basal cells with some progression towards differentiation into secretory cells). We then took steps towards development of methodologies to isolate and culture stem cells. In that year, we developed techniques to isolate viable single cells that retained cell surface antigens from digested fresh tissues, a prerequisite for using selective techniques to isolate stem cells; showed that a small population of cells expressed CD133, a putative prostate stem cell marker; and demonstrated successful infection with lentivirus of single cells from tissues, another tool that can be used to isolate cells expressing signaling pathways associated with “stemness”.

Based on our progress in Year Two, we expected in Year Three to return to our *in vivo* studies but with primary cultures that contained at least a subpopulation of stem cells. However, our progress in Year 3 was not as anticipated due to staffing problems. The research associate working on methodology to culture stem cells was on maternity leave from February to June, then the technician who carries out animal studies left in July. Therefore, we asked for and received a No-Cost Extension for an additional year, until January 14, 2008, to complete our studies. Our results are discussed below.

BODY

Our first designated task was to transplant primary epithelial cell cultures grown on OptiCell membranes under the renal capsule of nude mice (months 1-12). Our specific goals were to **(a)** prepare epithelial cell cultures on OptiCell membranes in vitro, **(b)** characterize epithelial cells grown on OptiCell membranes in vitro, **(c)** transplant epithelial cells grown on OptiCell membranes in vivo, and **(d)** characterize epithelial cells grown in vivo. We accomplished all components of this aim, as described in the first annual progress report (February, 2005). We concluded that the OptiCell membranes were not suitable as an implantation platform because they caused an undesirable inflammatory response. The membranes, composed of a proprietary plastic, are rather rigid and presumably cause physical trauma to the kidney, hence the inflammatory reaction and scar formation.

Our second aim was to transplant primary stromal cell cultures grown on OptiCell™ membranes under the renal capsule of nude mice (months 13-20). Our specific goals were to **(a)** prepare stromal cell cultures on OptiCell membranes in vitro, **(b)** characterize stromal cells grown on OptiCell membranes in vitro, **(c)** transplant stromal cells grown on OptiCell membranes in vivo, and **(d)** characterize stromal cells grown in vivo. Although our original intent had been to not initiate studies with stromal cells until Year Two, we carried out experiments in conjunction with the epithelial cells in Year One, and these results were also described in the first annual progress report. Histologic analysis of the implanted membranes revealed the same phenomenon as noted in the experiments with epithelial cells. Even the OptiCell membrane itself with no cells caused inflammation and extensive scar formation, as was also seen with the membranes carrying cells. This validated our conclusion from the previous studies that the membranes acted as an irritant in the kidney and this property precludes their utility as a platform for implantation of cell cultures.

Our third aim was to transplant co-cultures of epithelial and stromal cells on OptiCell™ membranes under the renal capsule of nude mice (months 21-36). Our specific goals were to **(a)** co-culture epithelial and stromal cells on OptiCell membranes in vitro, **(b)** characterize co-cultures of epithelial and stromal cells grown on OptiCell membranes in vitro, **(c)** transplant co-cultures of epithelial and stromal cells on OptiCell membranes in vivo, and **(d)** characterize co-cultures of epithelial and stromal cells in vivo. Although we had not planned to initiate co-culture experiments until Year 3, we decided to start some of these experiments since we were already working with epithelial and stromal cells in Aims 1 and 2. Carrying out this Aim required in vitro studies to first identify optimal co-culture conditions, which were described in the first annual progress report. We did not attempt to implant any co-cultures on OptiCell membranes into nude mice given the problem with inflammation that we encountered in Aims 1 and 2.

At the end of Year One, we concluded that OptiCell membranes would not provide a suitable platform for implantation of primary cultures of prostatic cells under the renal capsule of nude mice. We expected to devote Year Two to identifying and testing other substrates for implantation. However, as alluded to in the Introduction, we postponed those studies. Instead, we devoted Year Two to testing methodology for the primary culture of prostate cancer stem cells. Our results were described in the Year Two Progress Report (February, 2006) and are briefly recanted below:

(1) Search for presence of stem cells in primary cultures established according to standard methodology. We concluded that our primary cultures as routinely established did not have stem cells because they lacked the stem cell properties of infinite self-regeneration, were incapable of anchorage-independent growth, and did not contain any CD133-positive cells (a putative stem cell marker). We then initiated a series of experiments aimed at altering our traditional primary culture methodology in order to establish primary cultures containing stem cells.

(2) Isolation of single cells from human prostate cancer tissues. Stem cells are often identified by the expression of specific cell surface antigens and sorted by flow cytometry. Therefore, in order to culture stem cells, it will be necessary to culture single cells. Our standard protocol for establishment of primary cultures involves digestion of tissues to acini, but not to single cells, because we have found that single cells do not attach or grow well. Subsequently, we proceeded to optimize a protocol to generate a good single cell suspension from prostate cancer tissue. The optimal protocol that we developed involves a 2-4 hr digestion of minced tissue with medium containing high concentrations of collagenase I and hyaluronidase to release prostatic acini, and a short (5-10 min) digestion with 0.2% trypsin/0.2% EDTA to release single cells from the acini. We typically obtain an average of 1-2 x 10^5 prostatic cells/0.1g tissue. The trypsinization doesn't destroy cell surface antigens as shown by immunolabeling with antibody against epithelial cell-specific antigen (ESA).

(3) Evidence of CD133-positive cells from freshly digested tissues. CD133 is a promising prostate stem cell marker currently under investigation. We examined the expression of CD133 in single cells freshly dissociated from prostate cancer tissue using the protocol described above and flow cytometry. Single cells generated from a fresh cancer specimen after surgery were stained with Phycoerythrin (PE)-conjugated CD133 antibodies. A distinct rare population (2.2%) of $CD133^+$ cells existed in the tumor specimen. Compared to a normal tissue specimen, there was a more than 5-fold enrichment of $CD133^+$ cells in the cancer specimen, consistent with the hypothesis that CSCs arise from the dysregulation of self-renewal of normal stem cells and therefore CSCs are in a greater number than their normal counterparts. These results demonstrated that CD133 is expressed by a rare population of cells in prostate cancer, characteristic of stem cell markers. In vitro culture of the cells generated from fresh tissue in the standard serum-free medium that our lab uses for primary culture of prostatic epithelial cells resulted in a loss of $CD133^+$ cells. Similarly, $CD133^+$ cells were not detected by flow cytometry in several primary prostate epithelial cell cultures established previously in our lab.

(4) Expression of GFP in prostate cells by lentivirus infection. Wnt signaling through the canonical β-catenin pathway has been shown to regulate stem cell renewal in several tissues [2]. Activation of TCF4-driven gene expression has been shown to be a direct downstream target event of the activation of Wnt signaling pathway, which is important for the maintenance of stem cells. Therefore, it is conceivable that stem cells in the prostate may also require TCF4-mediated gene expression to maintain their stemness. To select cells that have activated TCF4, we used GFP as a reporter and delivered TCF4-GFP, a construct in which GFP is linked to a promoter with three TCF4 binding sites, into cells by lentivirus infection. We infected single cells generated from a prostate cancer specimen with lentivirus carrying either wild type TCF4-GFP or mutated TCF4-GFP, and analyzed the GFP expression using flow cytometry. Approximately 12% of the cells infected with wild type TCF4-GFP showed considerably higher level of GFP compared to cells infected with mutated TCF4-GFP, demonstrating the existence of a small population of cancer cells with activated TCF4-mediated gene expression. We also examined CD133 expression in these infected cells, and found that the differences in CD133 expression between isotype control and antibody-stained cells were minimal, consistent with our previous observation that in vitro culture under standard conditions resulted in a loss of CD133 expression in CSCs or a loss of CSCs all together.

(5) Primary culture of single cells. After demonstrating that we could create a population of viable single cells that retained cell surface antigens from digested fresh prostate cancers, our next goal was to identify conditions that would permit attachment and growth of these single cells *in vitro*. We first tested our standard primary culture conditions, which include collagen-coated dishes and the serum-free medium "Complete PFMR-4A" [3]. While these conditions are optimal for the attachment and growth of acini, single cells did not attach or grow in these conditions. We proceeded to test a number of other media and substrates and found success by using a feeder layer of stromal cells (mouse 3T3 cells) and a newly commercially available

defined medium from CellNTec (CnT-12).

(6) γc$^-$/RAG2$^-$ mice as an in vivo model for identification of CSCs. To obtain definitive evidence of the existence of CSCs, an *in vivo* functional analysis must be established. It is essential to determine whether a population of cells can initiate tumors *in vivo* in order to distinguish tumorigenic vs. non-tumorigenic cells. Various research groups have attempted to establish xenograft models of fresh, histologically intact human prostate cancer tissues in immunodeficient mice. The low engraftment rate experienced by these groups can be attributed to two main factors, the host environment and the grafting site. Several xenotransplantation models were developed based on severe combined immunodeficient (SCID) mice and their derivative, the non-obese diabetic (NOD)/SCID mouse model. The utility of the existing SCID mouse models is limited due to several disadvantages, including some "leakiness" that results in the appearance of mature B/T lymphocytes and immunoglobulins, residual natural killer (NK) cell activity, and a high rate of spontaneously developing thymomas that limit their lifespan. A new SCID mouse model has been developed by crossing mice lacking the common cytokine receptor _ chain for interleukin (IL)-2, IL-4, IL-7, IL-9, and IL-15 with mice lacking the recombinase activating gene-2 (RAG-2). The offspring has a stable phenotype characterized by the absence of all T and B cells and NK function. This novel immunodeficient mouse proved to be useful for studying xenotransplantation of human plasmacytoid dendritic cell precursors and human peripheral blood lymphocytes for the development of a severe acute graft-vs.-host disease model [4]. It also has been widely used by Dr. Weissman's lab and other stem cell investigators at Stanford with great success. The group has been astounded at the growth of enriched glioblastoma stem cells in this host. Glioblastomas, like prostate cancer, have traditionally proven very difficult to grow as xenografts in mice. Enriched stem-like cells from head and neck cancers, as well as from ovarian cancers, have also been growing well in the _c$^-$/RAG2$^-$ mice. We established our own breeding colony of these mice.

Progress in Year Three

(1) Optimization of cell sorting by flow cytometry. Since putative stem cells are often selected by expression of particular cell surface antigens (e.g., CD133) or signaling pathways (e.g. Wnt), it is imperative to be able to sort viable, rare cells from single cell populations obtained from freshly digested tissues. In Year Two, we found that we could isolate viable single cells from digested tissues that retained cell surface antigens or were infectable by lentiviruses. We found evidence of CD133-positive cells by FACS analysis and evidence of GFP-lentivirus expression driven by Wnt signaling. We also found that single cells could be cultured using a 3T3 feeder layer and CnT-12 medium. Next, we wanted to see if we could sort single cells by FACS that would retain viability and grow in culture. This proved to be a challenge. When we either sorted cells by CD133 expression or GFP expression after lentivirus infection, they did not grow in culture. Additional experiments showed that cells were not viable after sorting. We have learned from colleagues that stem/epithelial cells from solid tumors, as opposed to hematopoietic cells, are difficult to sort because they are very fragile. Over time, we have worked with the flow cytometer facility to modify nozzle size and flow force in an effort to cause less cell damage; optimization is still in progress. Other problems with flow cytometry have included contamination and difficulty in scheduling (because we never know exactly what time surgery will be completed). In an effort to avoid these problems, we also tested cell separation with antibody-conjugated magnetic beads, but we were dissatisfied with the inadequate separation of different cell populations.

(2) Cell isolation based on biological properties. Given our problems with cell isolation by FACS, we focused on other putative properties of stem cells. One of these is the ability to exclude the dye Hoescht 33342. This is the so-called "side population" of cells that express the ABCG2 transporter that actively excludes drugs and dyes. Since Hoescht 33342 is toxic, cells that can't exclude the dye

(non-stem cells) die, whereas the dye-excluding stem cells live. First, we used our standard primary cultures as examples of non-stem cells to determine their sensitivity to Hoescht 33342. Cells were treated with 0.5-5.0 μg/ml of the dye for 1 hour, then tested for their growth potential. The ID50 was ~1.5 μg/ml. Next, we isolated single cells from a digested cancer tissue and treated half of the cells with Hoescht 33342. The other half was untreated as a control. The cells were then inoculated onto 3T3 feeder layers in medium CnT-12. The dished inoculated with the treated cells (putatively containing dye-resistant stem cells) yielded a greater number of colonies than the untreated population (suggesting selection of stem cells with greater clonogenic potetial?). The dye-resistant colonies also had a different morphology from the untreated colonies. We repeated this experiment several times with cells from different cancer tissues, with similar results.

Progress in Year 4

In Year 4, we returned to in vivo studies. In order to improve our skills with the technique of subrenal implantation, one of the lab staff traveled to Vanderbilt University and took a training course from Dr. Simon Hayward, a recognized expert on this technique. The trainee also learned how to make androgen pellets and implant them into mice, since it is necessary to boost androgen levels of mice for successful growth of human prostate cells. Initially, we implanted cells from a primary culture established in the medium "CnT-12", which, as we discussed previously, is said to support the proliferation of stem/progenitor cells. The primary culture was derived from cancer tissue, and the cells were suspended in the extracellular matrix "Matrigel" prior to injection under the renal capsule. After one week, we recovered the implant and examined formalin-fixed sections after staining with H&E or after immunohistochemical labeling (Fig. 1). Staining with an antibody specific for human cells (Ku-70) showed that numerous human cells were present in the explant. However, although epithelial cells survived, clearly there was squamous differentiation, as shown by positive labeling with keratin 10.

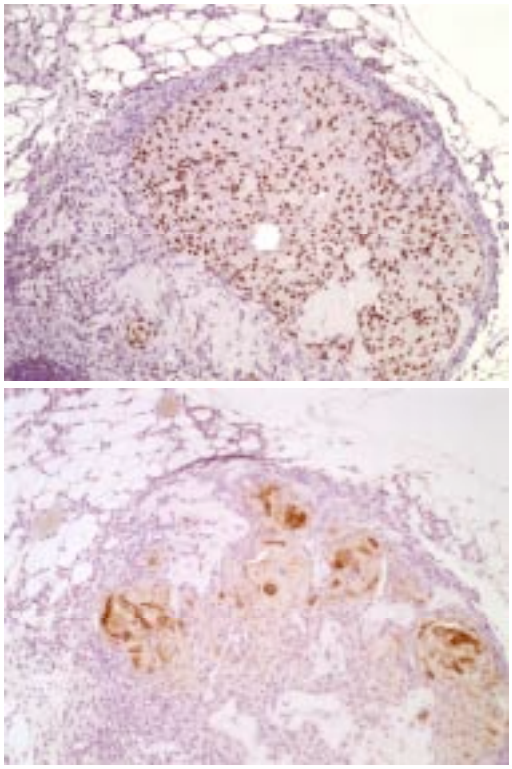

Fig. 1. Primary culture of epithelial cells implanted under the renal kidney of a mouse and recovered one week later. Top panel shows human cells stained brown with anti-human antibody Ku-70. Bottom panel shows keratin 10 staining in cells undergoing squamous differentiation.

Given these less than encouraging results, we decided to focus on a different type of in vitro/in vivo culture model that we have been developing. This is "tissue slice culture", in which cells are not isolated and grown in monolayer culture, but instead an intact slice of tissue is cultured. This technique was described in a review by Parrish et al. [5]. The advantage to tissue slice cultures is that cell:cell and cell:matrix interactions remain intact, and diverse histopathological elements (i.e., normal glands, cancer, etc.) may be represented in a given section. Furthermore, stromal:epithelial interactions, considered of utmost importance in the prostate, are sustained. We have carried out a number on in vitro studies with tissue slice cultures, one of which was published in PNAS [6]. Therefore, we extended our studies of tissue slice cultures to the in vivo situation. Cores of tissue, 5-mm in diameter, were obtained from normal or malignant prostate tissues. These were precision cut at 300-microns in a Krumdieck microtome, which is specially designed to cut fresh tissue. These were then implanted under the renal capsule of mice and recovered after various periods of time. Our studies so far suggest that both normal and malignant tissues can be maintained under the renal capsule of mice at least short-term (~ one week) (Fig. 2). The epithelium did not become squamous, and glandular structure appeared to be well-maintained. We will continue

KEY RESEARCH ACCOMPLISHMENTS

- Determined that OptiCell membranes do not provide a suitable substratum for implantation of prostate cells under the renal capsule of mice
- Developed methods to isolate and culture single cells from prostate tissues
- Tested a variety of approaches to isolate and culture prostate stem cells
- Determined that implantation under the renal capsule does not prevent squamous differentiation of primary cultures of prostate cells or cultures of putative stem cells
- Obtained preliminary evidence that tissue slice cultures might provide a suitable model system that can be maintained in vitro as well as under the renal capsule of mice.

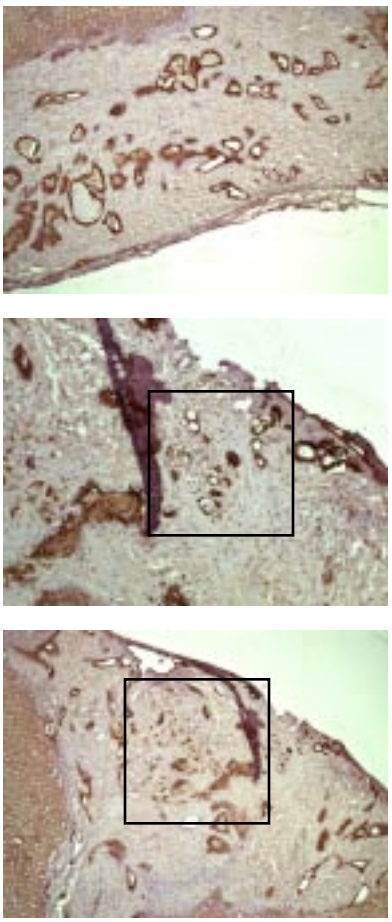

Fig. 2. Tissue slices maintained under the renal capsule of mice for one week show survival of normal tissue (top), grade 3 cancer (middle, in box) and grade 4 cancer (bottom, in box). Epithelia stained for keratin 18.

REPORTABLE OUTCOMES

None.

CONCLUSIONS

Our original premise was that primary cultures of prostate cancer cells would be capable of tumor formation *in vivo* if provided with the appropriate environment. We hypothesized that the appropriate environment would be under the renal capsule, where hypoxia would not be present and would not provoke growth-limiting, inappropriate squamous differentiation. We thought that the gas permeable membrane, "OptiCell", might provide the ideal substratum for implantation of primary cultures, but this turned out not to be the case. Due to their rigidity, OptiCell membranes provoked an inflammatory response and scarring. We also decided that, based on accumulating evidence, only stem cells might be capable of tumor formation under the renal capsule. We used a number of approaches to isolate and culture stem cells, but none definitively led to the isolation of stem cells. Such cells implanted under the renal capsule still became squamous. Finally, we chose to test another type of culture, "tissue slice culture", for its ability to survive and maintain structure and function under the renal capsule. Implantation of tissue slices under the renal capsule gave promising results. The cells in the tissues did not become squamous and structure of normal glands and cancer appeared to be preserved. We will continue to develop tissue slices as a promising in vitro/in vivo model of prostate cancer.

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APPENDICES

None.